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In Vitro Biological Activity of Prenylflavanones

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Abstract. *The biological activity of ten prenylflavanones purified from Sophora tomentosa L., and Sophora moorcroftiana Benth. ex Baker (Leguminosae) was investigated. The flavanones with prenyl-, lavandulyl- or geranyl groups on A ring, and two bioactive flavonostilbenes on ring B and stilbene (resveratrol) showed tumor-specific cytotoxic activity, antimicrobial activity, and anti-HIV activity, radical generation, and O_2^- scavenging activity. There was a positive relationship between radical generation and O_2^- scavenging activity in these prenylflavanones. These data suggest the medicinal significance of prenylflavanones.*

Some flavonoids such as flavones, isoflavones and calcones can modulate multidrug resistance (MDR) in cancer chemotherapy by binding to transmembrane P-glycoprotein (P-gp) which mediates anti-MDR action (1). By treatment with a flavonoid quercetin, the multidrug-resistant cells regained their sensitivity against adriamycin by inhibiting the ATP-binding site of P-gp (2). The binding affinity of flavonoids depended on both their class and substituents. Cytosolic P-gp flavonoid-binding site partly overlaps the ATP-binding site (3-6). We investigated here the biological activity of prenylflavanones, in conjunction with their radical modulating activity (determined by electron spin resonance (ESR) spectroscopy).

Sophora species have been used worldwide as folklore medicines and their constituents have been identified. Sofalcone, a flavonoid derived from *Sophora subprostrata* had strong anti-ulcer activity (7, 8), and modified the gastric blood flow (9, 10). Sophoraflavanone G (5,7,2',4'-tetrahydroxy-8-lavandulylflavanone) also from a *Sophora* inhibited *in vitro* the growth of oral bacteria including primary cariogenic mutant *Streptococci,* other oral *Streptococci, Actinomycetes,* and *Lactobacilli* (11).

In our studies on plant root components, we found that antioxidative activities of isoflavones from *Astragalus membranaceus* Bunge were superior or similar to those of butyl hydroxyltoluenes (12). The differences in antioxidant activities depended on the interaction between their chemical structures and reactive oxygen species (13). This data suggest that a radical generation by these isoflavones might be related to these antioxidative effects.

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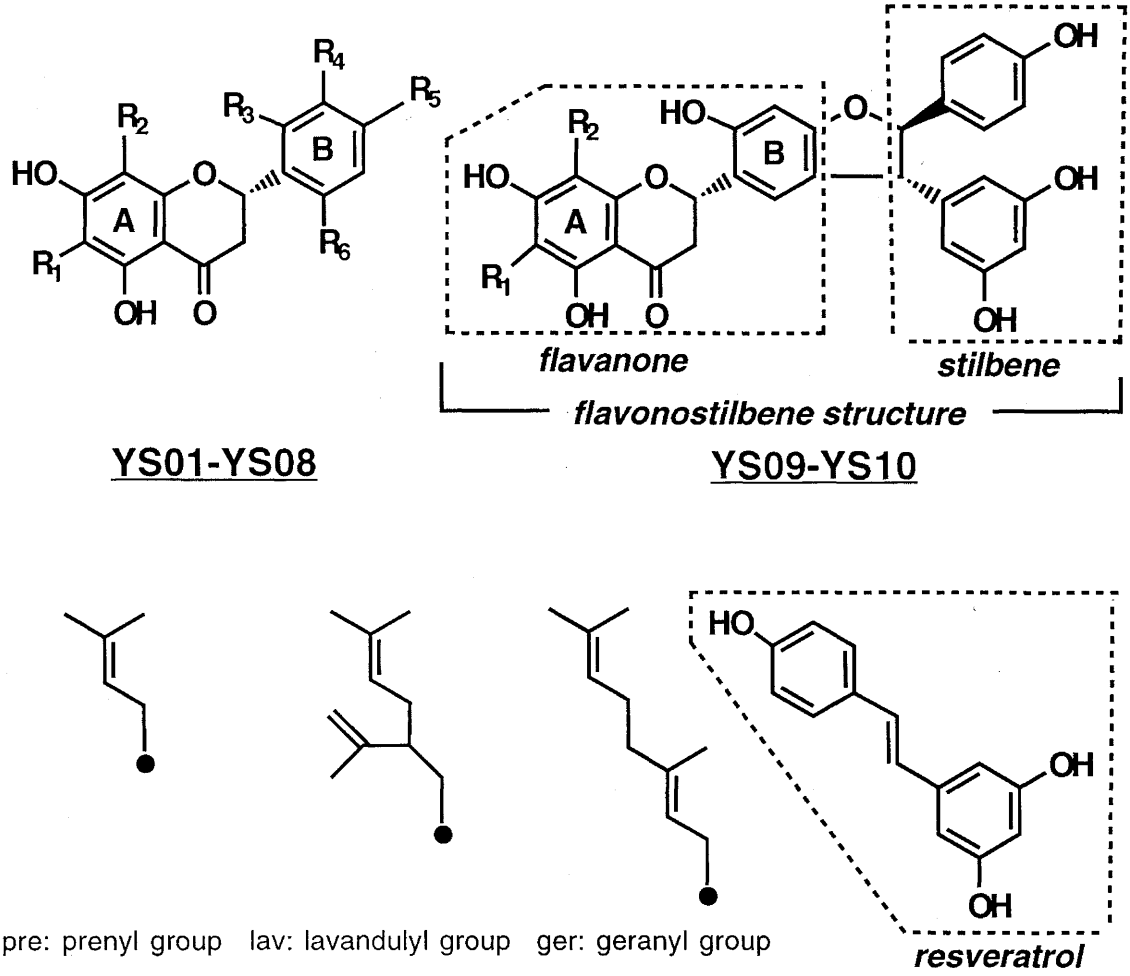
Key Words: Sophora species, prenylflavanones, cytotoxic activity, anti-HIV activity, antimicrobial activity, ESR, radical intensity, radical scavenging activity.

Materials and Methods

Materials. The following chemicals and reagents were obtained from the indicated companies: RPMI1640 medium, Dulbecco's modified Eagle medium (DMEM) (Gibco BRL, Grand Island, NY); fetal bovine serum (FBS) (JRH Biosci); 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Wako Pure Chem Ind., Ltd., Osaka); 3'-azido-2',3'-dideoxythymidine (AZT), dideoxycytidine (ddC) (Sigma); dextran sulfate (8 kD) (Kowa, Tokyo); diethylenetriaminepentaacetic acid (DETAPAC) (Sigma Chem. Co., St. Louis, MO); 3,4-dihydro-2,2-dimethyl-2*H*-pyrrole-1-oxide (DMPO) (a spin trap agent) (Aldrich Chemical Comp. In, U.S.A.); superoxide dismutase (SOD) from bovine erythrocytes (Dojin, Kumamoto, Japan). A strain of *Helicobacter pylori* (ATCC43504) was purchased from American Type Culture Collection (Rockville, MD).

Compounds. Naringenin (5,7,4'-trihydroxyflavanone) [**YS01**] and hesperetin (5,7,3'-trihydroxy-4'-methoxyflavanone) [**YS02**] were purchased from Sigma Chemical Company. St. Louis, MO, U.S.A. 6-Prenylnaringenin [**YS03**], sophoraflavanone B [**YS04**] (14, 15), sophoraflavanone A [**YS05**] (14, 16), sophoraflavanone G [**YS06**] (17), sophoraflavanone D [**YS07**] (18), euchrestaflavanone A [**YS08**] (14, 19), sophoraflavanone H [**YS09**] (20) and sophoraflavanone I [**YS10**] (20) were purified and characterized as described.

Assay for cytotoxic activity. Human squamous cell carcinoma (HSC-2) and human oral gingival fibroblasts (HGF) (5-7 population doubling levels) were cultured in DMEM medium supplemented with 10% heat-inactivated FBS. These cells were incubated for 24 hr with the indicated concentrations of test samples. The relative viable cell number (absor-



Compound	A ring		B ring			
	R_1	R_2	R_3	R_4	R_5	R_6
YS01	H	H	H	H	OH	H
YS02	H	H	H	OH	OMe	H
YS03	pre	H	H	H	OH	H
YS04	H	pre	H	H	OH	H
YS05	H	ger	H	H	OH	H
YS06	H	lav	OH	H	OH	H
YS07	ger	H	OH	H	OH	OH
YS08	H	pre	H	pre	OH	H
YS09	H	pre				
YS10	H	lav				

1) All compounds are analytically pure and soluble in DMSO.

Figure 1. *Structures of prenylflavanones [YS01-YS10].*

Table I. *Diverse biological activity of prenylflavanones [YS01-YS10].*

Compd	Cytotoxic activity[1] (CC_{50}:μg/mL) (mM) Human oral tumor cell line		Human gingival fibroblast (HGF)	Anti-HIV activity			Radical generation (at pH10.5)	O_2^- scavenging activity at pH7.8 (SOD U/1.5 mg)
	HSC-2	HSG		CC_{50} (μg/mL)	EC_{50} (μg/mL)	SI (CC_{50}/EC_{50})		
YS01	425 (1.560)	>500 (>1.840)	>500 (>1.840)	>200	>200	>>1	<0.03	1.5
YS02	370 (1.230)	480 (1.590)	189 (0.630)	>200	>200	>>1	<0.03	2.1
YS03	22 (0.065)	32 (0.094)	35 (0.103)	125	>200	<1	0.25	17.1
YS04	19 (0.056)	20 (0.059)	23 (0.068)	95	>200	<1	<0.03	3.9
YS05	<16 (<0.039)	16 (0.039)	13 (0.032)	21	>200	<1	<0.03	4.8
YS06	<8 (<0.019)	8 (0.019)	8 (0.019)	23	4	5	<0.03	5.6
YS07	24 (0.055)	58 (0.132)	45 (0.102)	113	>200	<1	<0.03	48.0
YS08	17 (0.042)	31 (0.076)	27 (0.066)	69	22	3	0.04	23.5
YS09	38 (0.065)	91 (0.156)	100 (0.172)	66	23	3	1.14	160.1
YS10	15 (0.023)	50 (0.077)	45 (0.069)	114	>200	<1	0.85	72.6
Dextran sulfate (μg/mL)				>1000	0.44	2273		
AZT (μM)				277	0.04	6925		
ddC (μM)				753	0.26	2896		

1) Near confluent HSC-2, HSG and HGF cells were incubated for 24 h with various concentrations of **YS01-YS10** and the relative viable cell number (A_{540}) was determined by MTT method. Each value represents mean from duplicate determinations. Control A_{540} values of HSC-2, HSG and HGF cells were 0.97, 0.54 and 0.31, respectively.

bance at 540 nm (A_{540})) was then determined by MTT assay. The 50% cytotoxic concentration (CC_{50}) was determined from the dose-response curve (21).

Assay for anti-human immunodeficiency virus (HIV) activity. Human T cell leukemia virus 1 (HTLV1)-bearing CD4 positive human T cell lines, MT-4 cells, were infected with HIV-1_{IIIB} at a multiplicity of infection (m.o.i.) of 0.01. HIV- or MOCK-infected MT-4 cells (1.5×10^5/mL, 200 μL) were placed into 96-well microtiter plates and incubated in the presence of varying concentrations of the fractions. After incubation for 5 days at 37°C in a 5% CO_2 incubator, cell viability was quantified by a colorimetric assay (at 540 nm and 690 nm), monitoring the ability of viable cells to reduce MTT to a blue formazan product. All data represent the mean values of triplicate measurements. CC_{50} was determined with MOCK-infected cells, whereas EC_{50} was determined with HIV-infected cells. Selectivity index (SI) was defined as follows: $SI=CC_{50}/EC_{50}$ (22).

Assay for radical intensity. Radical intensity was determined at 25°C using ESR spectroscopy (JEOL JES RE1X, X-band, 100 kHz modulation frequency). Instrument settings: center field, 335.6 ± 5.0 mT; microwave power, 8 mW; modulation amplitude, 0.1 mT; gain, 630 time constant, 0.1 sec; scanning time, 2 min. Radical intensity was determined in 0.1M $NaHCO_3/Na_2CO_3$ buffer (pH 10.5) containing 50% DMSO. The final concentration of compound was 1.5 mg/mL and the radical intensity was defined as the ratio of peak heights of these radicals to that of MnO (23).

Superoxide anion (O_2^-) scavenging activity. O_2^- was generated by hypoxanthine (HX) and xanthine oxidase (XOD) reaction (200 μL) [2 mM HX (in 0.1 M PBS, pH 7.8) 50 μL, 0.5 mM DETAPAC 20 μL, DMPO 10 μL, sample (in DMSO) 50 μL, H_2O or SOD 30 μL, XOD (0.5 U/mL in 0.1M PBS) 40 μL]. The gain was changed to 250. O_2^- scavenging activity was expressed as SOD unit/1.5 mg sample (24).

Antimicrobial activity. Ten compounds were investigated for antimicrobial activity against fourteen bacteria. The test was carried out by serial dilutions of the compound in nutrient agar (Oxoid) base. *Staphylococcus aureus* 6571, 8530, 8531, *Staphylococcus typhimurium* 4, 57, 59, *Bacillus subtilis* VB1, *Shigella dysenteriae* 1, *Shigella sonnei* 2, *Escherichia coli* Row, *Escherichia coli* R832, *Klebsiella spp* 14, *Providencia spp* 1, and *Vibrio cholerae* 865 from clinical isolates were obtained from Central Laboratory of Clinical Microbiology, University of Calcutta, India (25).

Anti-Helicobacter pylori activity. A strain of *Helicobacter pylori* (ATCC43504) was purchased from American Type Culture Collection (Rockville, MD). Mueller-Hilton broth containing 5% FBS was used as the medium, and was cultured in a jar conditioned with Campylo Pack (Dia Iatron) for 48 hours. Briefly, *H. pylori* strains were inoculated on a

Table II. *Antimicrobial activity of prenylflavanones [YS01-YS10].*

Compd	Gram-positive bacteria							Gram-negative bacteria							*Helicobacter pylori*
	Staphylococcus aureus			*Staphylococcus typhimurium*			*Bacillus subtilis* VB1	*Shigella*		*Escherichia coli*		*Klebsiella spp* 14	*Providencia spp* 1	*Vibrio cholerae* 865	
	6571	8530	8531	4	57	59		*dysenteriae 1*	*sonnei 2*	ROW	R832				MIC_{50} (μg/mL)[1)]
YS01	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100.0
YS02	25	100	100	>100	>100	50	>100	>100	>100	50	>100	>100	50	100	>100.0
YS03	25	50	50	>100	>100	>100	>100	50	>100	>100	25	>100	>100	25	>100.0
YS04	50	25	25	>100	>100	>100	>100	50	>100	>100	12.5	>100	>100	25	68.0
YS05	25	25	25	>100	>100	>100	>100	25	>100	>100	25	>100	>100	50	>100.0
YS06	12.5	12.5	12.5	>100	>100	>100	>100	12.5	>100	>100	12.5	>100	>100	12.5	5.8
YS07	25	12.5	>100	>100	>100	>100	>100	25	>100	>100	>100	>100	>100	100	21.0
YS08	12.5	12.5	>100	>100	>100	>100	>100	25	>100	>100	>100	>100	>100	>100	>100.0
YS09	25	12.5	>100	>100	>100	>100	>100	50	>100	>100	>100	>100	>100	>100	2.6
YS10	12.5	25	>100	>100	>100	>100	>100	12.5	>100	>100	>100	>100	>100	>100	2.1
Clarithromycin															10.0

1) The MIC value (μg/mL) was determined for each compound by a broth microdilution method using *H. pylori* (ATCC43504) (refs. 26 and 27). Each **YS01** and **YS02:** each equivqlent mixture of 2*R* and 2*S*.

Brucella agar plate containing 10% horse serum, and cultured at 37°C for 48 hours. The bacterial colonies collected were diluted to 10^7 colony forming unit (CFU)/mL with 0.9% saline. The extracts were dissolved in DMSO, and then diluted with Mueller-Hilton broth. To the solution of the extracts, each bacterial suspension was added to a density of 10^6 colony forming units (CFU)/100 mL/well. The mixture was incubated at 37°C for 48 hours. The minimum inhibitory concentration (MIC) of each fraction was calculated from the dose-response curve (26, 27).

Results

Cytotoxic activity. Among ten prenylflavanones, four compounds [**YS04, YS05, YS06, YS08**] showed higher cytotoxic activity against both two oral tumor cell lines (HSC-2, HSG) (Table I). HSG cells were more resistant than HSC-2 cells. In particular, **YS09** and **YS10** showed higher cytotoxic activity against tumor cells (HSC-2, HSG) than against normal human gingival fibroblast (HGF) (Table I).

Anti-human immunodeficiency virus (HIV) activity. A compound **YS06** showed the highest anti-HIV activity (Selectivity Index (SI=5), followed by **YS08** (SI=3) and **YS09** (SI=3) (Table I). The other seven compounds were inactive (SI=1).

Radical generation and O_2^- scavenging activity. Among ten prenylflavanones, flavonostilbenes of [**YS09, YS10**] produced stronger ESR signal of radical at pH10.5 than other seven compounds, followed by **YS03**. The other compounds did not produce any detectable amount of radical. **YS09** (160.1 SOD unit/1.5 mg) showed the highest O_2^- scavenging activity, followed by **YS10** (72.6 SOD unit), **YS07** (48.0 SOD unit), **YS08** (23.5 SOD unit), **YS03** (17.1 SOD unit) and **YS06** (5.6 SOD unit) (Figure 2) (Table I).

Antimicrobial activity. **YS06** (10 μg/mL) showed the highest antibacterial activity against three strains of *Staphylococcus aureus* 6571, 8530 and 8531, followed by **YS08, YS09** and **YS10** (Table II). **YS06** was also effective against *Shigella dysenteriae* 1, *E. coli* R832 and *Vibrio cholerae* 865.

YS10 showed the highest anti-*H. pylori* activity, followed by **YS09** and **YS06** (Table II).

Discussion

We found that two prenylflavanones [**YS09, YS10**] of a flavonostilbene type with the skeletons of flavanone on B ring and stilbene (resveratrol) showed remarkable properties,

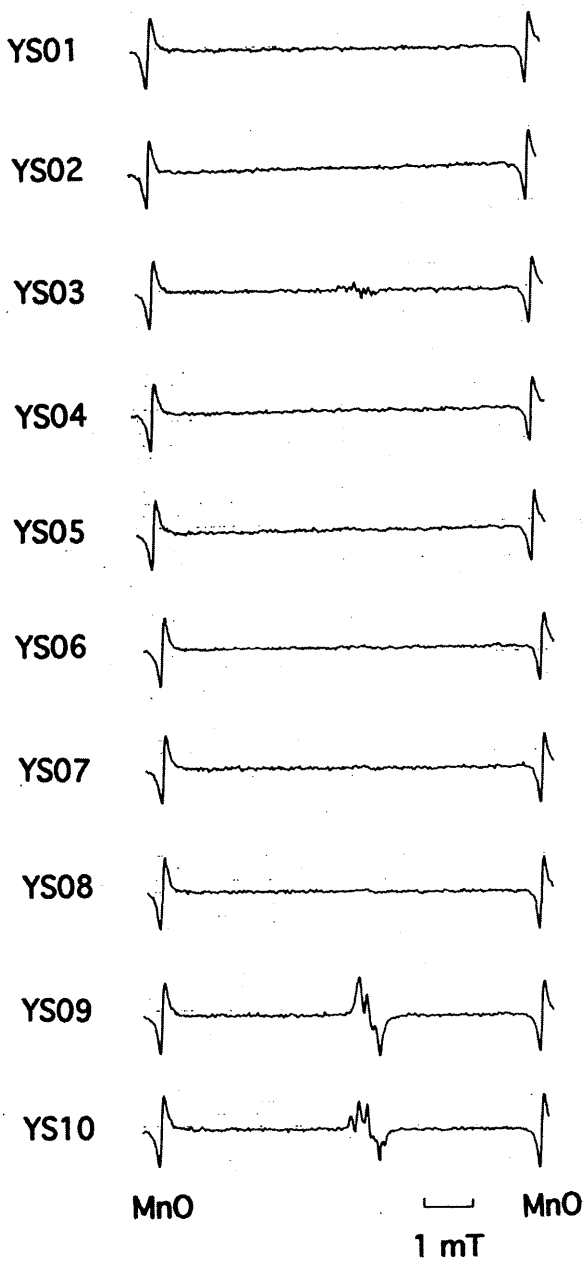


Figure 2. *ESR spectra of prenylflavanones [YS01-YS10]. Samples were applied to ESR spectroscopy 1 min after being dissolved in 0.1 M $NaHCO_3/Na_2CO_3$ buffer (pH10.5) containing 50% DMSO.*

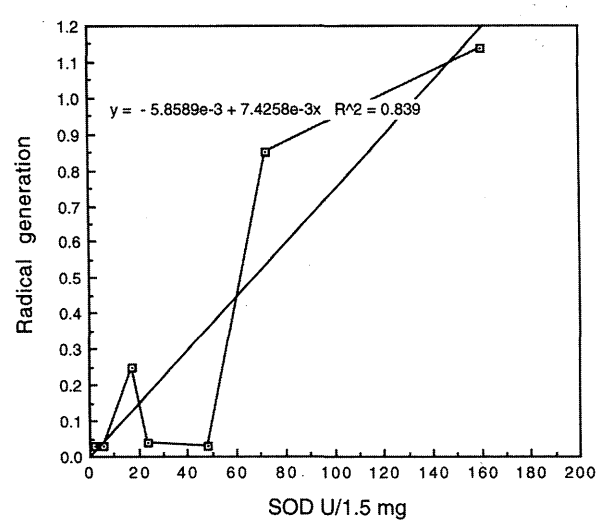


Figure 3. *Relationship between radical generation (at pH 10.5) and O_2^- scavenging (SOD) activity (at pH 7.8).*

possibly due to their cell membrane affinity (28) (Table I). These compounds showed selective cytotoxicity against tumor cells than against normal cells, produced higher amounts of radical (Figure 2), and showed the highest O_2^- scavenging activity.

When the O_2^- scavenging activity of ten prenylflavanones was plotted *vs.* SOD activity, there was a positive correlation between these parameters (an equation in Figure 3). Introduction of lavandulyl group at A ring [**YS06, YS10**] enhanced significantly these activities, whereas substitution by hydrogen at R_1 and R_2 on A ring [**YS01, YS02**] considerably reduced the biological activity. We have recently found that polyprenylalcohols such as geranylgeraniol and geranylfarnesol neither produced any radical nor scavenged O_2^-. This data suggest that many compounds display both radical generation and O_2^- scavenging activity.

We found that three compounds [**YS06, YS08, YS09**] showed the highest anti-HIV activity (SI=3-5.0) among ten compounds. These compounds have either lavandulyl- or prenyl group at position R_1 of A ring. This suggests that both hydrophobic groups (lavandulyl- or prenyl group) on A ring and hydrophilic group (hydroxyl group) on B ring in the same structure might markedly enhance their binding affinity with cell membrane. Especially, two lavandulyl- and hydroxyl groups such as **YS06** (SI=5) might be important determinants of the anti-HIV activity.

We found that **YS06** (10 mg/mL) showed the highest antibacterial activity among ten compounds against three *Staphylococcus aureus, Shigella dysenteriae* 1, *E. coli* R832, and *Vibrio cholerae* 865, followed by two flavonostilbenes [**YS09, YS10**] (Table II). Previous reports have demonstrated the efficacy of **YS06** against other bacterial strains (11). On the other hand, flavonostilbenes [**YS09, YS10**] were more effective against *H. pylori*, as compared with **YS06.**

The present study demonstrated several interesting properties of two flavonostilbenes (sophoraflavanone H [**YS09**], sophoraflavanone I [**YS10**]): tumor specific-cytotoxic activity, antimicrobial activity, anti-HIV activity, radical generation and O_2^- scavenging activity. There remains to investigate the mechanism by which **YS09** and **YS10** induce

these effects. We have recently found that isoprenoid-substituted flavonoids induced apoptotic cell death (29), more potently than tannin-related compounds (30). Further screening of flavonoids, especially those which have isoprenoid side chains, is under way.

Acknowledgements

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